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Cytokine-Like Activities of a Humoral Opsonin from the Solitary Urochordate *Styela Clava*

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ABSTRACT—Previously, we have identified and purified an opsonin from the plasma of the solitary urochordate, *Styela clava*. Here we show that this opsonin exhibits cytokine-like activies in tunicates. The opsonin enhances incorporation of ^{3}H-thymidine into cultures of pharyngeal tissues from *S. clava* and acts as a powerful chemoattractant. Cytokine-like activity is also indicated by the ability of the *S. clava* molecule to activate tunicate phagocytes. In addition, we show that the opsonin is produced by *S. clava* hemocytes and that its production is stimulated by zymosan. These data suggest that the opsonin characterized here is identical to a tunicate, cytokine-like molecule (tunIL-1β) previously identified by its ability to stimulate mouse thymocyte proliferation.

INTRODUCTION

Opsonins play a key role in invertebrate humoral defense systems. We have previously identified a humoral opsonin in the tunicate, *Styela clava* [8]. Our studies indicate that preincubation of target cells wih *Styela clava* plasma increases the capacity of tunicate hemocytes for phagocytosis. Opsonization is inhibited by the carbohydrates mannan, N-acetyl-D-galactosamine, and galactose, and by the divalent cation chelator, EDTA. Such data suggest that the *S. clava* hemolymph may contain a C-type lectin [6]. This lectin-like activity has recently been isolated from *S. clava* hemolymph [9]. Gel filtration yielded a fraction with strong opsonic activity that was associated with a single, electrophoretically-resolved protein. The fractionated opsonin is sensitive to tryptic digestion and heat denaturation, and its biological activity is dose-dependent. Molecular characterization indicated that the opsonic protein is a 17.5 kDa monomer with a pI of 7.0.

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We postulate that the humoral opsonin from *S. clava* may subserve multiple inducible biological activities during inflammatory reactions. Such a diversity of function is typical of inflammatory proteins in mammals. For instance, the mammalian inflammatory cytokine, IL-1, has multiple activities including the enhancement of IL-2 and IL-2 receptor expression by T-cells, the induction of slow wave sleep, increased thymocyte and fibroblast proliferation and the regulation of chemotaxis and hematopoiesis [5]. IL-1 also appears to be involved with communication between the immune and nervous systems [3, 4].

Similarly, complement components in mammals subserve diverse effector functions. C3 has the capacity to opsonize foreign targets, initiate the subsequent lytic cascade of complement components, release an anaphylatoxin that is both chemoattractive and stimulatory and is involved in the activation of phagocytes and lymphocytes [7].

The propensity of inflammatory protein in mammals to have multiple biological activities may also be reflected in tunicates. Here we test a purified opsonin from *S. clava* for its ability to stimulate cell proliferation, chemotaxis, and cell activation.

MATERIALS AND METHODS

Tunicates

Styela clava was purchased from Marinus Inc., Long Beach, CA. Tunicates were maintained at 15°C in an aerated, sandbed-filtered, 180-liter aquarium filled with artificial sea water (Instant Ocean, Aquarium Systems, Mentor, OH; 3.4% w/v). Approximately 2 ml of Marine Invertebrate Diet (Carolina Biological, Gladstone, OR) were added to the aquarium every two days. Tunicates were allowed to adjust to conditions in the aquarium for one week prior to experimentation.

Hemocyte Suspensions and Plasma

Tunicates were bled by severing the stolon and collecting 1.5 ml of the exuding hemolymph in chilled, polystyrene, centrifuge tubes containing 1.5 ml of sterile-filtered, artificial sea water (ASW) (Instant Ocean; 3.4% w/v). The resulting hemocyte suspensions were allowed to stand for five minutes so that large debris and cell aggregates sank. The upper 2 ml of each suspension were then transferred to fresh tubes. The number of hemocytes in suspension was determined and the volume adjusted with ASW. Hemocytes from individual tunicates were not pooled for any assay.

Plasma was obtained by harvesting hemolymph in the absence of ASW. Cells and debris were removed by centrifugation ($800\times g$, 15 minutes, twice) and the resulting plasma stored frozen (−20°C).

Proliferation of Pharyngeal Explants

Tunicate Tissue Culture Medium: Tunicate tissue culture medium (T-RPMI) was prepared in sterile-filtered ASW and contained, per liter, 454 mg RPMI 1650 powder (with L-glutamine, without sodium bicarbonate; Sigma Chemicals, St. Lous, MO), 10^5 units penicillin sulfate (Sigma Chemicals), and 100 mg streptomycin sulfate (Sigma Chemicals) [10]. This medium was then sterilized by filtration and stored at 4°C.

Tunicate Tissue Culture Protocol: The stiulatory effects of purified opsonin were tested in cultures of pharyngeal tissue from *S. clava*. Pharyngeal tissue is considered to be a hematopoietic field in tunicates thereby providing an excellent source to study cell proliferation. The procedure used to culture pharyngeal explants was similar to that of Raftos *et al.* [10]. Pharyngeal tissue was diced into $3\times3\times1$ mm^3 explants and rinsed three times in T-RPMI. Rinsed explants were transferred to 96-well-flat-bottomed culture plates (1 explant/well; Costar, Pleasanton, CA) contianing T-RPMI (200 μl/well). Explants were cultured at 15°C in normal atmosphere.

Incubation of Tunicate Explants with Purified Opsonin: Explants were equilibrated to culture conditions for 3 days prior to adding purified opsonin. After this period, explants were transferred to either fresh T-RPMI or T-RPMI containing 1 μg/ml of purified opsonin (200 μl/well) [9]. Explants were cultured at 15°C in normal atmosphere for a further 3 days.

Quantification of ^{3}H-thymidine Uptake: Explants were removed from tissue culture plates and pooled in 2 ml of the same medium in which they had been cultured. Tissues were then incubated at 15°C with 5 μCi/ml methyl ^{3}H-thymidine (^{3}H-TdR, 20 Ci/mmol; ICN Radiochemicals, Costa Mesa, CA). After 18 hr, excess ^{3}H-TdR was removed by washing the explants three times (1 hr per wash) in 4 ml ASW on a hematology mixer (Fisher Scientific, Pittsburgh, PA). Wahsed explants were transferred to scintillation vials (1 explant/vial) containing 200 μl trypsin (2% w/v in ASW) and digested overnight at 37°C. Two milliliters of scintillation cocktail (Ecolite; ICN Radiochemicals) was added to each vial so that the incorporated radioactivity could be quantified with a Beckman LS 3150P Scintillation Counter. Data are presented as proliferation indexes (P.I.) such that:

P.I. = (counts per minute in experimental trials)/(counts per minute in controls).

Mean values for each trial were calculated from P.I.s for individual trials.

Hemocyte Migration Assay

The migration of tunicate hemocytes was quantified using chemotaxis chambers in which Transwell inserts (6.5mm diameter, 5.0 μm pore size polycarbonate filter, tissue culture treated; Milli-

pore, Bedford, MA) and 24-well tissue culture plates (Costar) formed the upper and lower wells respectively. One hundred microliter aliquots of cell suspensions (2×10^6 cells/ml) were added to the upper wells of the chemotaxis chambers. When required, purified opsonin was added to a final concentration of 1.0 μg/ml to the upper well immediately after the addition of cells. The lower well was filled with 600 μl ASW with or without purified opsonin (1 μg/ml). Cells were allowed to migrate through Transwell membranes for 2 hr (15°C). After incubation, the chemotaxis chambers were agitated vigorously, the Transwell inserts removed, and the plates centrifuged ($200\times g$, 5 min. 4°C). Cells that had migrated to the lower wells were counted in 16 randomly selected 400× fields of view using an inverted (tissue culture) microscope. Data are presented as migration indexes (M.I.) such that:

M.I. = (cells per field of view in experimental trials)/(cells per field of view in controls).

Mean values for each trial were calculated from M.I.s for individual trials.

Zymosan Stimulation of Opsonin Production

Zymosan-treated Hemocyte Cultures: Two hundred microliters of *S. clava* hemocyte suspensions (2×10^6 hemocytes/ml in ASW) were added to 48-well tissue culture plates. The plates were centrifuged ($200\times g$, 5 min.) and the supernatant removed and replaced with 200 μl either T-RPMI or zymosan (50 μg/ml in T-RPMI). The cells were incubated 48 hr at 15°C. Following incubation, the plates were centrifuged ($300\times g$, 5 min.) and the supernatants removed and pooled. Pooled supernatants were centrifuged ($500\times g$, 10 min.) to remove cells and particulate zymosan and then filter-sterilized (0.22 μm; Costar).

Assays of Supernatants: Culture supernatants were analyzed in two ways. First, they were tested for opsonic acitivity as described previously by Kelly, *et al.* [9]. Second, the supernatants were concentrated thirty-fold using Centriprep 10 centrifugal concentration units (Amicon, Danvers, MA) and then assayed for protein content by the Bradford method (Protein Determination Kit, Biorad, Richmond, CA) and subjected to SDS-PAGE (see below).

Activation of Phagocytes by Purified Opsonin

Pre-incubation of Latex Beads: 800 μl aliquots of latex bead (Sigma LB 30, Sigma Chemicals) suspensions were washed ($16{,}000\times g$, 6 min) and resuspended in 200 μl of ASW or purified opsonin. The beads were incubated at room temperature with constant agitation for 1.5 hr. Suspensions were then washed thrice ($16{,}000\times g$, 6 min) and resuspended in ASW to a final concentration of 5×10^6 beads/ml.

Phagocytosis of Latex Beads: Cover glasses (22 mm×22 mm, Gold Seal, Fisher Scientific) were suspended between moistened strips of filter paper in glass petri dishes. Each cover slip was overlaid with 100 μl of hemocyte suspension (3×10^6 cells/ml). Hemocytes were allowed to adhere to cover slips for 2 hr (15°C). Cover slips were then washed thrice (800 μl ASW), and 50 μl of purified opsonin (500 ng/ml) or ASW were added. Cultures were allowed to incubate for 30 min (15°C) before being overlaid with 50 μl of pre-incubated latex beads (5×10^6 beads/ml). These cultures were incubated for a further 30 min at 15°C before excess latex beads were removed by dipping each cover slip in ASW ten times. The cells were then fixed in 200 μl of absolute methanol (15 min, 4°C) and washed in distilled water. Cover slips were inverted onto microsope slides and sealed.

Quantification of Phagocytosis: Phagocytosis was assessed by phase contrast microscopy (1,250 ×magnification, oil immersion). A minimum of 200 hemocytes from at least four fields of view per cover slip were inspected. Data were recorded as the percentage of hemocytes that had phagocytosed any number of latex beads (% phagocytic cells) relative to the total cell population. Data are presented as phagocytic stimulation indexes (PSI) where:

PSI = (% phagocytic cells in experimental trials)/(phagocytic cells in controls).

Mean values for each trial were calculated from PSIs for individual trials.

SDS-PAGE

SDS-PAGE was performed according to the

method of Laemmli [1] using 12% separating gels. Dalton VII-L markers (Sigma Chemicals) were used for calibration. *Styela clava* plasma (total protein concentration 70 μl/ml) and G-50 gel filtration-purifed opsonin (49 μg protein/ml) [9] were diluted 1:1 in 2×SDS/sample buffer with 2% v/v 2-mercapoethanol while tunIL-1β (200 μg protein/ml) [2] and zymosan-stimulated supernatant (260 μg protein/ml) were diluted 1:4 in sample buffer. Gels were silver-stained using an AG-5 kit according to the manufacture's instructions (Sigma Chemicals).

Purification of Opsonin and TunIL-1β

The procedures used to purify opsonin have been described in detail elsewhere [9]. Thirty milliliters of concentrated tunicate plasma was subjected to gel filtration. Plasma was sterilized by filtration (0.22 μm) and concentrated thirty-fold using Centriprep 10 centrifugal concentration units (Amicon). Concentrated plasma was then applied to a gel filtration chromatography column (bed volume 150 ml) packed with Sephadex G-50 (Sigma Chemicals) in phosphate-buffered saline. Hemolymph fractions were eluted with PBS, and fractions of 2.5 ml were collected and immediately sterilized by filtration. The hemolymph fractions were assessed for opsonic activity, and the purity of the opsonic fraction was confirmed by a single band on silver-stained 12% SDS-PAGE gel.

The procedures used to purify tunIL-1β have also been described in detail elsewhere [2]. Tunicate IL-1-like proteins were isolated from 100 ml of tunicate hemolymph. After concentration by ultrafiltration (PM 10 membrane, Amicon, Danvers, MA), hemolymph was applied to a Biogel AcA 54 gel filtration column (Pharmacia, Piscataway, NJ) which was eluted with phosphate buffered saline. Fractions were assayed for IL-1 activity in a mouse thymocyte proliferation assay. Peaks of IL-1 activity were pooled, concentrated and applied to a PBE 84 chromatofocusing column (Pharmacia). The chromatofocusing column was eluted with a linear pH gradient (pH 8.4–pH 4.0) and fractions were assayed for IL-1-like activity as above. Two predominant species, one being tunIL-1β, were isolated. The purity of both species was confirmed on silver-stained 12% SDS-PAGE gels.

Statistical Analysis

Statistical analyses were performed with the *Mystat* software package (Systat, Inc., Evanston, IL). The statistical significance of differences between mean values was determined by the Student's *t*-test [14]. Differences were considered to be significant for probabilities of less than 5.0%.

RESULTS

Effect of Purified Opsonin on ^{3}H-thymidine Uptake

Incubation of pharyngeal explants from *S. clava* with purified opsonin resulted in a significant ($P<0.05$) increase in the level of ^{3}H-thymidine uptake when compared to controls incubated with T-RPMI (Fig. 1). Pharyngeal explants which had been cultured for three days with purified opsonin (1 μg/ml in T-RPMI) followed by 18 hr with ^{3}H-thymidine (5 μCi/ml) exhibited a proliferation index of 39.8±4.8.

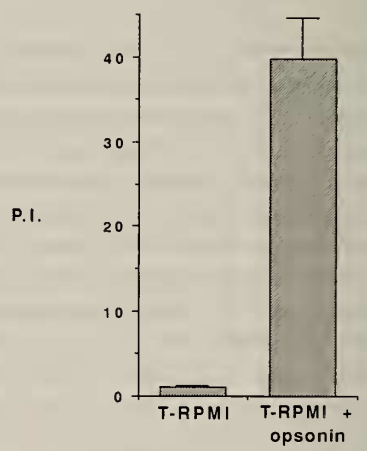

Fig. 1. Proliferation indexes (±SEM, n=20 explants) measured by ^{3}H-thymidine uptake for *S. clava* pharyngeal explants incubated in the presence or absence of purified *S. clava* opsonin.

Purified Opsonin Enhances Hemocyte Migration

When added to the lower wells of chemotaxis chambers, purified *S. clava* opsonin significantly ($P<0.05$) increased the number of tunicate hemocytes migrating through Transwell membranes

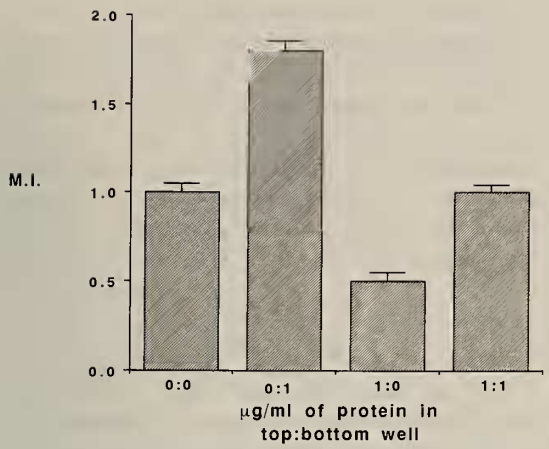

FIG. 2. Migration indexes (±SEM, n=5) for hemocytes incubated in Transwell chambers containing either ASW in both the upper and lower wells, ASW in the upper well and 1 μg/ml *S. clava* opsonin (in ASW) in the lower well, 1 μg/ml *S. clava* opsonin (in ASW) in the upper well and ASW in the lower well, and 1 μg/ml *S. clava* opsonin (in ASW) in both the upper and lower wells.

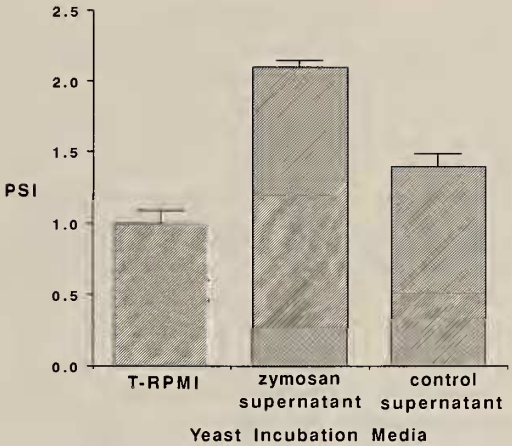

FIG. 3. Phagocytic stimulation indexes (±SEM, n=6) for hemocytes exposed to yeast that had been pre-incubated with either T-RPMI, zymosan-stimulated hemocyte supernatant, or supernatant from cells cultured in the absence of zymosan (control supernatant).

(Fig. 2). Approximately twice as many cells (migration index 1.8±0.05) entered lower wells containing 1 μg/ml of opsonin relative to controls containing ASW alone (migration index 1.0± 0.05). A reciprocal pattern was evident when purified opsonin was added to the upper wells of chemotaxis chambers. The addition of 1 μg/ml of opsonin to the upper wells reduced migration by 50% (migration index 0.5±0.05) relative to ASW-controls ($P<0.05$). The addition of purified opsonin to both the upper and lower wellls ($1\times g$/ml) yielded no significant change ($P>0.05$) in the number of tunicate hemocytes migrating through the Transwell membranes (migration index 1.0± 0.04) compared to ASW-controls.

Effect of Zymosan-stimulated Hemocyte Supernatants on Phagocytosis

Incubation of yeast in supernatants from zymosan-stimulated hemocyte cultures significantly ($P<0.05$) increased their phagocytosis by tunicate hemocytes when compared to yeast incubated in T-RPMI alone (Fig. 3). The phagocytic stimulation index for zymosan-stimulated-hemocyte supernatants was twice (PSI=2.1±0.05) that of T-RPMI controls (PSI=1.0±0.09). Incubation of yeast in supernatants of hemocyte cultures that had not been exposed to zymosan also increased phagocytosis (PSI=1.4±0.09) relative to T-RPMI controls ($P<0.05$). However, this level was signi-

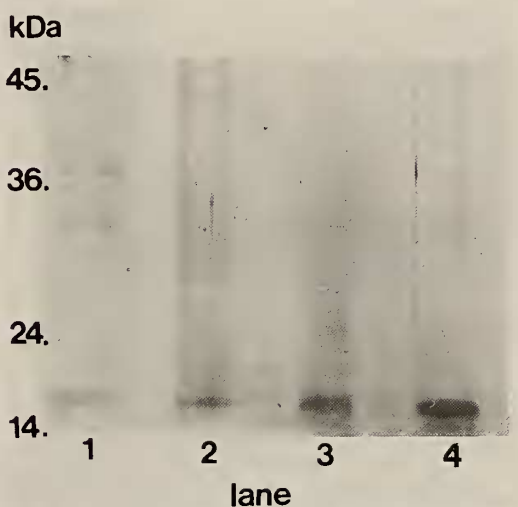

FIG. 4. Silver-stained, reducing SDS-PAGE of *Styela clava* plasma (lane 1), zymosan-stimulated hemocyte supernatant (lane 2), tun-IL 1β (lane 3), and G-50 column purified opsonin from *S. clava* plasma (lane 4). Position of molecular weight markers are shown at the left (kDa).

ficantly lower ($P<0.05$) than that for the phagocytosis of yeast which had been opsonized by zymosan-stimulated hemocyte supernatants.

Characterization of Zymosan-stimulated Hemocyte Supernatant

SDS-PAGE of zymosan-stimulated hemocyte supernatant resolved a number of proteins with a prominent band at 17.5 kDa under reducing conditions (Fig. 4, lane 2). A similar 17.5 kDa protein was evident in whole *S. clava* plasma (Fig. 4, lane 1). The major protein evident in zymosan-stimulated hemocyte supernatant migrated to the same position as both purified *S. clava* opsonin and tunIL-1β (Fig. 4, lanes 4 and 3 respectively).

Activation of Phagocytes by Purified S. clava Opsonin

Pre-incubation of hemocytes with purified *S. clava* opsonin significantly ($P<0.05$) increased their capacity to phagocytose latex beads when compared to hemocytes pre-incubated in ASW (Fig. 5). Hemocytes which had been incubated with purified opsonin had a PSI of 3.0 ± 0.22 in the presence of ASW-incubated latex beads while control (ASW-incubated) hemocytes exhibited a PSI of 1.0 ± 0.15. The addition of latex beads pre-incubated in purified opsonin to ASW-incubated hemocytes did not significantly ($P>0.05$) alter the phagocytic stimulation indexes (1.2 ± 0.14) when compared to controls comprised of ASW-incubated hemocytes exposed to ASW-incubated latex beads.

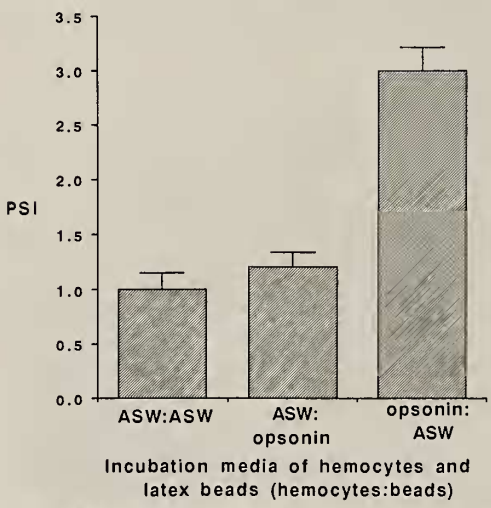

Fig. 5. Phagocytic stimulation indexes ($\pm$SEM, n=5) for tunicate hemocytes exposed to latex beads under the following conditions: ASW-incubated cells with ASW-incubated beads (ASW:ASW), opsonin-incubated cells with ASW-incubated beads (opsonin:ASW), ASW-incubated cells with opsonin-incubated beads (ASW:opsonin). Hemocytes were cultured 30 minutes at 15°C with either ASW or opsonin before the addition of latex beads.

DISCUSSION

We have shown that an opsonin from *Styela clava* exhibits several functional characteristics which are typical of cytokine-like molecules. First, the *S. clava* opsonin stimulates the proliferation of tunicate cells. Differences in cell proliferation between control and experimental cultures of *S. clava* pharyngeal tissue are readily quantified by uptake of ^{3}H-thymidine [11, 12]. Raftos *et al.* [13] have shown that ^{3}H-thymidine incorporation by cultured explants in inhibited by irradiation and competition with non-isotopic thymidine. This suggests that there is a direct relationship between cell proliferation and ^{3}H-thymidine uptake [12]. Hence, the enhanced incorporation of ^{3}H-thymidine in pharyngeal cultures containing *S. clava* opsonin indicates that this molecule can regulate the proliferation of *S. clava* cells *in vitro*.

Purified opsonin also acts as a chemoattractant for *S. clava* hemocytes (Fig. 2). The addition of purified opsonin to the lower well of chemotaxis chambers significantly increased the level of migration of hemocytes to the lower well. Conversely, when opsonin was added to the upper well of the chamber, migration to the lower well was inhibited while no net movement was evident when equal concentrations of opsonin were present in both the upper and lower wells of the chemotaxis chambers. This type of analysis indicates that the *S. clava* opsonin acts as a powerful chemoattractant for tunicate hemocytes.

In addition to its proliferative and chemotactic activities, the *S. clava* molecule activates tunicate phagocytes. Pre-incubation of *S. clava* hemocytes with purified opsonin enchanced the level of phagocytosis of latex beads. This enhanced phagocytic activity cannot be explained by the opsonization

of latex beads by the *S. clava* protein. The *S. clava* protein acts as a carbohydrate specific lectin [8] which, as shown here, is not opsonic for abiotic targets such as latex. Latex beads incubated with opsonin are not ingested at a greater rate than that of untreated controls. Hence, the increased phagocytosis of unopsonized latex beads by hemocytes in the presence of *S. clava* opsonin must reflect the ability of this protein to metabolically activate tunicate hemocytes.

The capacity of *S. clava* opsonin to regulate cellular processes such as proliferation, chemotaxis, and hemocyte activation indicates that it may play a central role in adaptive inflammatory reactions. However, adaptive reactions such as chemotaxis would be dependent upon the differential production of opsonin. To fulfill that prerequisite, we have shown that the secretion of *S. clava* opsonin is induced by antigenic challenge. Assays of supernatants from hemocytes cultured with zymosan revealed an enhanced secretion of opsonic molecules relative to controls cultured in the absence of zymosan. SDS-PAGE indicated that this induced opsonic activity was associated with a 17.5 kDa protein that is of identical electrophoretic mobility to purified *S. clava* opsonin. It is reasonable to conclude that the *S. clava* opsonin is produced by hemocytes in repsonse to stimulation by the target, zymosan.

The functional and physiochemical properties identified here suggest *S. clava* opsonin is identical to a cytokine-like protein, designated tunIL-1β, that has been isolated from the same species [2, 11]. Both the opsonin studied here and tunIL-1β are 17.5 kDa proteins with pI's of 7.0–7.4 [9, 11]. Both molecules are opsonic [13], and exhibit similar sensitivity to inhibition by N-acetyl-D-galactosamine, galactose, and mannose [8]. They enhanced the proliferation of pharyngeal explants *in vitro* above the normal levels [11], act as chemoattractants (D.A. Raftos, personal communication), and activate phagocytic cells [13]. Moreover, production of both *S. clava* opsonin and tunIL-1β by *S. clava* hemocytes is stimulated by zymosan [13]. These data suggest that tunicates express an opsonic protein with cytokine-like activities which can mediate adaptive inflammatory responses.

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